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(21) International Application Number: PCT/US93/00889 (22) International Filing Date: 2 February 1993 (02.02.93) (30) Priority data: 832,236 7 February 1992 (07.02.92) US (71) Applicant: THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; National Institutes of Health, Office of Technology Transfer, Box OTT, Bethesda, MD 20892-9902 (US). (72) Inventors: LEVINE, Rodney, L. ; 1502 Auburn Avenue, Rockville, MD 20850 (US). KARLSTROM, Anders, R. ; Strandbergsgatan 49, S-112 87 Stockholm (SE). SHAMES, Brian, D. ; 6A Talcott Forest Road, Farmington, CT 06032 (US).		(74) Agents: KILYK, John, Jr. et al.; Leydig, Voit & Mayer; Two Prudential Plaza, Suite 4900, Chicago, IL 60601-6780 (US). (81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: USE OF 5,5'-DITHIO-BIS(2-NITROBENZOIC ACID) FOR INHIBITION OF HIV PROTEASE (57) Abstract A method and composition for inhibiting the growth or replication of a virus, such as a retrovirus, in particular a human immunodeficiency virus, specifically HIV-1, through reaction with the viral protease on an exposed surface, in particular an exposed surface outside of the active site of the viral protease, which method preferably involves contacting the virus with a composition comprising a sulfhydryl-reactive compound, such as 5,5'-dithio-bis(2-nitrobenzoic acid).		

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Use of 5,5'-dithiobis(2-nitrobenzoic acid) for inhibition of hiv protease.

TECHNICAL FIELD OF THE INVENTION

This invention relates to a method of inhibiting the growth or replication of viruses. In particular, this invention relates to a method of inhibiting viral proteases, especially HIV-1 protease. This invention also relates to a composition which comprises a sulfhydryl-reactive compound, such as 5,5'-dithio-bis(2-nitrobenzoic acid), useful in inhibiting the growth or replication of viruses, such as the HIV-1 virus.

BACKGROUND OF THE INVENTION

Acquired immune deficiency syndrome (AIDS) is a very serious disease, reported cases of which have increased dramatically within the past several years. Estimates of reported cases in the very near future also continue to rise dramatically. Consequently, there is a great effort to develop drugs and vaccines to combat AIDS.

The AIDS virus was first identified in 1983. It has been known by several names and acronyms. It is the third known T-lymphocyte virus (HTLV-III), and it has the capacity to replicate within cells of the immune system, causing profound cell destruction. The AIDS virus is a retrovirus, a virus that uses reverse transcriptase during replication. This particular retrovirus is also known as lymphadenopathy-associated virus (LAV), AIDS-related virus (ARV) and, most recently, as human immunodeficiency virus (HIV). Two distinct types of HIV have been described to date,

namely HIV-1 and HIV-2. The acronym HIV will be used herein to refer to HIV viruses generically.

Specifically, HIV is known to exert a profound cytopathic effect on the CD4+ helper/inducer T-cells, thereby severely compromising the immune system. HIV infection also results in neurological deterioration and, ultimately, in the death of the infected individual.

The field of viral chemotherapeutics has developed in response to the need for agents effective against retroviruses, in particular HIV. There are many ways in which an agent can exhibit anti-retroviral activity. For example, HIV requires at least four viral proteins for replication: reverse transcriptase (RT), protease, transactivator protein (TAT), and regulator of virion-protein expression (REV). Accordingly, viral replication could theoretically be inhibited through inhibition of any one or all of the proteins involved in viral replication.

Anti-retroviral agents, such as AZT and ddC, are known to inhibit RT. There also exist anti-viral agents that inhibit TAT.

A useful approach being investigated recently for potential use in the treatment of AIDS is the development of synthetic peptides as inhibitors of the retroviral protease. It is known that retroviruses, including HIV, express their genetic content by directing the synthesis of a polyprotein by the host. The polyprotein is a precursor molecule, which is processed through proteolysis to generate essential viral enzymes and structural proteins. The virally encoded protease is contained within the polyprotein

and is responsible for cleaving the polyprotein to yield mature viral proteins.

Since the protease is known to be required for viral replication, it has been a therapeutic target for the development of AIDS drugs. Drug development programs aimed at the protease are generally focused on the synthesis of inhibitors that bind to the active site of the protease. The compounds are generally peptide analogues with chemical modifications that prevent the protease from cleaving the viral polyprotein to yield mature viral proteins. Some of these inhibitors are peptides or peptide analogues that were originally studied as inhibitors of structurally related proteases, such as pepsin and renin. Such an approach has generated over 50 potent inhibitors of the protease. Several of these inhibitors are scheduled for clinical trials.

Although these inhibitors are effective in preventing the protease from functioning in the proteolysis of the polyprotein, the inhibitors suffer from some distinct disadvantages. First of all, since the active site of the protease is hindered, i.e., has reduced accessibility as compared to the remainder of the protease, the ability of the inhibitors to access and bind in the active site of the protease is impaired. Secondly, the peptide inhibitors that bind to the active site of the protease are generally poorly soluble in water, causing distinct problems in drug delivery. Given the fact that the ultimate goal is the development of pharmaceutical compositions suitable for therapeutic treatment of the disease, such problems in drug delivery limit the utility of these inhibitors.

An inhibitor of the viral protease that functions at a site other than the active site of the enzyme would be desirable. Such an inhibitor would offer distinct advantages over presently developed inhibitors in that the inhibitor would not be hindered by reduced accessibility or inaccessibility of its site of action, unlike an inhibitor that functions in the active site of the protease. Moreover, an inhibitor that functions at a site other than the active site of the viral protease would enable the development of useful, water-soluble pharmaceutical formulations.

BRIEF SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method of inhibiting the growth or replication of a virus, such as a retrovirus, in particular a human immunodeficiency virus, specifically HIV-1.

It is another object of the present invention to provide a method of inhibiting a viral protease, such as a retroviral protease, in particular a human immunodeficiency virus, specifically the HIV-1 protease.

It is still another object of the present invention to provide a composition, in particular a pharmaceutical composition, which is preferably topically administered and which inhibits the growth or replication of a virus, such as a retrovirus, in particular a human immunodeficiency virus, specifically HIV-1.

It is an additional object of the present invention to provide a composition, in particular a

pharmaceutical composition, which may be topically administered and which inhibits a viral protease, such as a retroviral protease, in particular a human immunodeficiency viral protease, specifically the HIV-1 protease.

Yet another object of the present invention is to provide a composition, in particular a pharmaceutical composition, which is preferably topically applied and which prevents infection of an animal, in particular a human, with a virus, such as a retrovirus, in particular a human immunodeficiency virus, specifically HIV-1.

A further object of the present invention is to provide a method of treating an animal, in particular a human, infected with a virus, such as a retrovirus, in particular a human immunodeficiency virus, specifically HIV-1.

A still further object of the present invention is to provide a method of treating an animal, in particular a human, to prevent infection with a virus, such as a retrovirus, in particular a human immunodeficiency virus, specifically HIV-1.

An even further object of the present invention is to provide a method of reversibly inhibiting the growth or replication of a virus under laboratory conditions for research purposes.

These and other objects and advantages of the present invention, as well as additional inventive features, will become apparent from the description herein.

The present invention provides a method of, and composition for, inhibiting the growth or replication

of a virus, particularly a retrovirus, specifically a human immunodeficiency virus such as HIV-1. In particular, the present invention provides for inhibiting a viral protease by contacting the virus with a composition which comprises a sulfhydryl-reactive compound that reacts with the viral protease so as to inhibit the growth or replication of the virus. The present invention is expected to have utility in the therapeutic treatment of a human infected with a virus, particularly a retrovirus, specifically a human immunodeficiency virus such as HIV-1, and in the prophylactic treatment of a human to prevent viral infection.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides a method of, and composition for, inhibiting the growth or replication of a virus, particularly a retrovirus, specifically a human immunodeficiency virus such as HIV-1. It has now been discovered that the growth or replication of a virus, particularly a retrovirus, specifically a human immunodeficiency virus such as HIV-1, can be inhibited by reaction with the viral protease at an exposed surface of the viral protease, e.g., at an exposed site outside of the active site of the viral protease. In particular, the inhibition of viral growth or replication has been effected by a reaction of a sulfhydryl-reactive compound with a sulfhydryl group-containing amino acid of the viral protease.

The present inventive method comprises contacting a virus with a composition comprising an inhibitory compound that will react with the viral protease,

thereby inhibiting the ability of the protease to cleave the viral polyprotein into its constituent proteins. It is preferred that the inhibitory compound be one which reacts with an amino acid that is on an exposed surface of the protease, most preferably an exposed surface outside of the active site of the enzyme. An exposed surface is one that is in free contact with the environment, thereby being readily accessible to an inhibitory compound of the present invention. Such ease of accessibility increases the effectiveness of the inhibitory compound and enables the formulation of water-soluble pharmaceutical compositions comprising the inhibitory compound.

Sulfhydryl-reactive compounds are preferred compounds for use in the context of the present invention. Sulfhydryl-reactive compounds are compounds that react with sulfhydryl groups. The preferred sulfhydryl-reactive compounds for use in conjunction with the present invention include 5,5'-dithio-bis(2-nitrobenzoic acid), which is more commonly referred to as DTNB or Ellman's reagent, as well as pharmaceutically effective salts and analogs thereof. (Ellman, Archives of Biochemistry and Biophysics 82:70-77 (1959)).

Ellman's reagent or DTNB has been used to quantitate sulfhydryl groups in proteins and biological tissues, and as a specific label for cysteine residues. (Riddles et al. Methods in Enzymology 91:49-60 (1983)). DTNB has been shown to inhibit the enzymatic activity of pyroglutamate aminopeptidase (Prasad et al., Brain Res. 364(2):331-337 (1986)), the porcine young adult isozyme of type III hexokinase (Kearse et al., Am. J.

Physiol. 249 (6 Part 2):R740-R746 (1985)), the extracellular protease of Serratia marcescens (Choi et al., Korean Biochem. J. 19(3):287-293 (1986)), human placental dipeptidylaminopeptidase III (Shimamori et al., Chem. Pharm. Bull. (Tokyo) 34(8):3333-3340 (1986)), and porcine IRCM-serine protease 1 (Cromlish et al., J. Biol. Chem. 261(23):10850-10858 (1986)).

It has been discovered that a sulfhydryl-reactive compound, such as DTNB, is capable of inhibiting a viral protease by effecting a reaction between the sulfhydryl-reactive compound and a sulfhydryl group in a sulfhydryl-containing amino acid, such as cysteine, in the viral protease. The sulfhydryl-reactive compound is believed to form a mixed disulfide between the sulfhydryl-reactive compound and the sulfhydryl group of the amino acid in the viral protease. This reaction inhibits the ability of the viral protease to cleave the polyprotein into the necessary viral proteins. This inhibition arrests viral growth or replication.

Sulfhydryl-reactive compounds inhibit HIV-1 by reaction with Cys-67 of the HIV-1 protease. However, it is believed that the histidine residue in position 69 also may be inhibited by reaction with an inhibitory compound so as to effect inhibition of the HIV-1 protease and arrest HIV viral growth or replication. Although there are no cysteine residues in the HIV-2 protease, it is believed that inhibition of similarly positioned amino acids may also result in its inhibition as well.

The present inventive method may be used to treat a virally infected animal, such as a human. The

present invention has particular usefulness in inhibiting the growth or replication of viruses, which are dependent on the action of a viral protease that contains a sulfhydryl group, such as viral proteases containing cysteine. Accordingly, the present invention is particularly well suited to treating animals infected with a retrovirus, such as a human immunodeficiency virus, specifically HIV-1, which is dependent on the action of a sulfhydryl group-containing viral protease for growth and replication.

The present inventive methods and compositions are expected also to have utility in the treatment of various non-retroviruses, in addition to retroviruses and human immunodeficiency viruses, such as HIV-1. Examples of possible viruses that may be suitable for treatment using the present invention include Type C and Type D retroviruses, HTLV-1, HTLV-2, FLV, SIV, MLV, BLV, BIV, equine infectious viruses, anemia viruses, avian sarcoma viruses, hepatitis type A, B, non A and non B viruses, herpes viruses, cytomegalo viruses, influenza viruses, arboviruses, varicella viruses, measles, mumps, and rubella viruses. Of these viruses, those that utilize cysteine-containing viral enzymes, in particular proteases, are expected to be treatable in accordance with the present invention.

Compositions comprising inhibitory compounds for use in the present inventive method preferably comprise pharmaceutically acceptable carriers in addition to the inhibitory compound. Pharmaceutically acceptable carriers are well-known to those who are skilled in the art, as are suitable methods of administration. The choice of carrier will be determined in part by the

inhibitory compound, as well as by the particular method used to administer the composition.

One skilled in the art will appreciate that various routes of administering a drug are available and, although more than one route may be used to administer a particular drug, a particular route may provide a more immediate and more effective reaction than another route. Furthermore, one skilled in the art will appreciate that the particular pharmaceutical carrier employed will depend, in part, upon the particular compound employed and the chosen route of administration. Accordingly, there is a wide variety of suitable formulations of the composition of the present invention.

Formulations suitable for oral administration may consist of liquid solutions, such as an effective amount of the compound dissolved in diluents, such as water, saline, or orange juice; capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as solids or granules; solutions or suspensions in an aqueous liquid; and oil-in-water emulsions or water-in-oil emulsions. Tablet forms may include one or more of lactose, microcrystalline cellulose, colloidal silicon dioxide, croscarmellose sodium, magnesium stearate, stearic acid, and other excipients, colorants, and pharmacologically compatible carriers.

Formulations suitable for topical administration include lozenges comprising the active ingredient in a flavor, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin, or sucrose and

acacia; and mouthwashes comprising the active ingredient in a suitable liquid carrier; as well as creams, emulsions, gels, and the like containing, in addition to the active ingredient, such carriers as are
5 known in the art.

Formulations for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Formulations suitable for vaginal administration
10 may be presented as pessaries, tampons, creams, gels, pastes, foams, or spray formulas containing, in addition to the active ingredient, such carriers as are known in the art to be appropriate. Similarly, the active ingredient may be combined with a lubricant as
15 a coating on a condom.

Formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which may contain anti-oxidants, buffers, bacteriostats, and solutes that
20 render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions that may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose sealed containers, such as
25 ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared
30 from sterile powders, granules, and tablets of the kind previously described.

The dose administered to an animal, particularly a human, in the context of the present invention should be sufficient to effect a prophylactic or therapeutic response in the infected individual over a reasonable time frame. The dose will be determined by the strength of the particular inhibitory compound employed, the severity of the disease state, as well as the body weight and age of the infected individual. The size of the dose also will be determined by the existence of any adverse side effects that may accompany the particular compound employed. It is always desirable, whenever possible, to keep adverse side effects to a minimum.

In the treatment of some virally infected individuals, it may be desirable to utilize a "mega-dosing" regimen, wherein a large dose of the inhibitory compound is administered, time is allowed for the compound to act, and then a suitable reagent is administered to the individual to inactivate the inhibitory compound, such as mercaprol (or BAL) and N-acetyl-cysteine, which inhibit sulfhydryl-reactive compounds.

The pharmaceutical composition may contain other pharmaceuticals in conjunction with the inhibitory compound, when used to therapeutically treat acquired immunodeficiency syndrome (AIDS). Representative examples of these additional pharmaceuticals include antiviral compounds, immunomodulators, immunostimulants, and antibiotics. Exemplative antiviral compounds include AZT, ddI, ddC, gancyclovir, and fluorinated dideoxynucleotides. Exemplative immunomodulators and immunostimulants include various

interleukins, CD4, cytokines, antibody preparations, blood transfusions, and cell transfusions. Exemplative antibiotics include antifungal agents, antibacterial agents, and anti-pneumocystis carinii agents.

5 Administration of the inhibitory compound with other anti-retroviral agents and particularly with known reverse transcriptase (RT) inhibitors, such as ddC, AZT, ddI, ddA, or other inhibitors that act against other HIV proteins, such as anti-TAT agents,
10 will generally inhibit most or all replicative stages of the viral life cycle. The dosages of ddC and AZT used in AIDS or ARC patients have been published. A virustatic range of ddC is generally between 0.05 μ M to 1.0 μ M. A range of about 0.005-0.25 mg/kg body weight
15 is virustatic in most patients. The preliminary dose ranges for oral administration are somewhat broader, for example 0.001 to 0.25 mg/kg given in one or more doses at intervals of 2, 4, 6, 8, 12, etc. hours. Currently 0.01 mg/kg body weight ddC given every 8
20 hours is preferred. When given in combined therapy, the anti-RT compound, for example, may be given at the same time as the inhibitory compound or the dosing may be staggered as desired. The two drugs also may be combined in a composition. Doses of each may be less
25 when used in combination than when either is used alone.

The use of some sulfhydryl-reactive compounds in accordance with the present inventive method offers the added advantage of reversibility. A reducing agent,
30 such as dithiothreitol (DTT), may be used to reverse the effect of the sulfhydryl-reactive compound or the growth or replication of a virus, such as the

retrovirus HIV-1. Specifically, DTT is believed to reduce the mixed disulfide bond formed between a sulfhydryl-reactive compound and a sulfhydryl group of an amino acid of the viral protease, thereby restoring the original sulfhydryl groups of the viral protease essential for viral growth or replication. This reversibility is of particular usefulness in the basic in vitro research of viral functions and in "mega-dosing" therapeutic regimens of virally infected individuals, in which case an antidote or a reversing reagent, such as a sulfhydryl reagent, may be used to "rescue" the individual from the effect of the "mega-dose." In these situations, where reversibility is not desired, a reducing agent or a sulfhydryl reagent is not used after a reversible sulfhydryl-reactive compound or, rather, an irreversible sulfhydryl compound is used.

The present inventive method and composition are further described in the context of the following examples. These examples serve to further illustrate the present invention and are not intended to limit the scope of the invention.

EXAMPLE 1

This example demonstrates the inhibition of recombinant HIV-1 protease with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB).

The HIV-1 aspartyl protease was contacted with DTNB. DTNB is a sulfhydryl-reactive compound, i.e., it reacts with sulfhydryl groups. There are two sulfhydryl groups in the HIV-1 aspartyl protease, namely in the two cysteine residues Cys-67 and Cys-95.

The Cys-67 residue is located on an exposed surface of the enzyme and is, therefore, readily accessible to DTNB. The Cys-95 residue is located in or very near where the two subunits of the protease bind to each other. This residue is, therefore, less accessible to DTNB. (Navia et al., Nature 337:615-620 (1989), and Wlodawer et al., Science 245:616-621 (1989)).

The DTNB reacted with the sulfhydryl groups of the two cysteine residues to form disulfide bridges between DTNB and each of the two cysteine residues. As a result of this reaction, the viral protease was inhibited from cleaving the viral polyprotein into its constituent proteins.

It was uncertain from this experiment, however, which of the two cysteine reactions was actually critical to the inhibition. Accordingly, a variant of the protease was studied. Specifically, a fusion protein was formed by fusing together the protease with an IgG binding domain (ZZ) at the amino terminus of the protease. (Boutelje et al., Archives of Biochemistry and Biophysics 283:141-149 (1990)). Essentially, this fusion protein has a long extension on its amino terminal side. It was reasoned, based on the three-dimensional structure of the protease, that the Cys-95 residue of the fusion protein would be shielded from reacting with DTNB. This would enable the determination of which cysteine residue is critical for inhibition of the protease.

The fusion protein was reacted with DTNB at pH 6.2 under non-reducing conditions and subsequently dialyzed into 20 mM HCl. (Sliwowski et al., Analytical Biochemistry 147:369-373 (1985)). When assayed for

activity at pH 5.5, the fusion protein was inactive. A control sample, which was treated in the same manner but was not exposed to DTNB, was fully active.

Pepsin digestion of the DTNB-reacted fusion protein revealed that only the Cys-67 residue was fully derivatized. The Cys-95 residue was only partially derivatized. These results indicated that Cys-67 was selectively derivatized, and its reaction with DTNB was responsible for the inhibition of the protease activity. These results further indicated that a chemical reaction with an amino acid on an exposed surface of the protease, in particular the reaction of a sulfhydryl-reactive compound, such as DTNB, with a surface cysteine residue, can inhibit a viral protease, in particular the HIV-1 protease, thereby inhibiting viral growth or replication.

EXAMPLE 2

This example demonstrates the reversion of the DTNB-inhibition of recombinant HIV-1 protease with dithiothreitol (DTT).

Exposure of the DTNB-reacted fusion protein of Example 1 with DTT for 5 minutes restored the activity of the viral protease to 70% of the control protease. DTT is a known reducing agent and is believed to have destroyed the disulfide bridges between DTNB and each of the cysteine residues by restoring their sulfhydryl groups. Accordingly, DTT reversed the effect of DTNB and enabled the restoration of the activity of the viral protease.

Although irreversible inhibitory compounds, such as irreversible sulfhydryl-reactive compounds, may be

used in accordance with the present invention, this example demonstrates the potential utility of a reversible inhibitory compound, such as a reversible sulfhydryl-reactive compound, in basic in vitro research of viral functions and in "mega-dose" therapeutic regimens, which may be used in the therapeutic treatment of virally infected humans.

EXAMPLE 3

This example sets forth a proposed therapeutic treatment of an HIV-1-infected human.

A pharmaceutical composition comprising a sulfhydryl-reactive compound in a pharmaceutically acceptable carrier is prepared. The pharmaceutical composition is then administered to an HIV-1-infected human in a therapeutically effective dose in a therapeutically effective regimen to inhibit further replication or growth of the HIV-1 virus.

The particular dose administered is dependent upon the severity of the disease state, the strength of the particular compound employed, and the age and body weight of the infected individual, as well as the route of administration. The dosage amount can be determined by routine experimentation and by reference, in part, to available information regarding other anti-HIV compounds and clinical trials.

Depending on the advanced state of viral infection, a "mega-dose" therapeutic regimen may be utilized to treat the individual, in conjunction with an antidote or reversing agent after a suitable period of elapsed time of treatment with a pharmaceutical composition comprising the active inhibitory compound.

The dosage amount of the inhibitory compound, the elapsed time of treatment with such an inhibitory compound, and the dosage of the antidote or reversing agent can be determined by routine experimentation and
5 by reference, in part, to available information regarding other anti-HIV compounds and clinical trials.

All publications referenced herein are hereby incorporated by reference in their entireties.

While this invention has been described with
10 emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that the preferred methods and compositions may be varied. It is intended that the invention may be practiced otherwise than as specifically described herein.

15 Accordingly, this invention includes all modifications encompassed within the spirit and scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of inhibiting the growth or replication of a virus, which method comprises contacting a virus with a composition comprising a
5 sulfhydryl-reactive compound.

2. The method of claim 1, wherein the virus encodes a viral protease comprised of at least one amino acid comprising a sulfhydryl group.

3. The method of claim 2, wherein said
10 sulfhydryl-reactive compound reacts with said sulfhydryl group of said viral protease.

4. The method of claim 3, wherein said sulfhydryl group is on an exposed surface of the viral protease.

5. The method of claim 4, wherein said exposed
15 surface is outside of the viral protease's active site.

6. The method of claim 3, wherein said amino acid is cysteine.

7. The method of claim 1, wherein said
20 sulfhydryl-reactive compound is selected from the group consisting of 5,5'-dithio-bis(2-nitrobenzoic acid) and pharmaceutically acceptable salts thereof.

8. The method of claim 7, wherein said
25 sulfhydryl-reactive compound is 5,5'-dithio-bis(2-nitrobenzoic acid).

9. The method of claim 1, wherein said virus is a retrovirus.

10. The method of claim 9, wherein said virus is a human immunodeficiency virus.

5 11. The method of claim 10, wherein said human immunodeficiency virus is HIV-1.

12. The method of claim 3, wherein said inhibition of viral growth or replication is irreversible.

10 13. The method of claim 3, wherein said inhibition of viral growth or replication is reversed by contacting said virus with a composition comprising a reducing compound that restores said sulfhydryl group of said viral protease.

15 14. The method of claim 13, wherein said sulfhydryl-reactive compound is 5,5'-dithio-bis(2-nitrobenzoic acid) and said reducing compound is dithiothreitol.

20 15. A method of treating a virus-infected animal, which method comprises administering to the animal a therapeutically effective dose of a pharmaceutical composition comprising a sulfhydryl-reactive compound.

16. The method of claim 15, wherein said animal is a human.

17. The method of claim 16, wherein said human is infected with a retrovirus.

18. The method of claim 17, wherein said human is infected with a human immunodeficiency virus.

5 19. The method of claim 18, wherein said human immunodeficiency virus is HIV-1.

20. The method of claim 15, wherein said
10 sulfhydryl-reactive compound is selected from the group consisting of 5,5'-dithio-bis(2-nitrobenzoic acid) and pharmaceutically acceptable salts thereof.

21. The method of claim 20, wherein said sulfhydryl-reactive compound is 5,5'-dithio-bis(2-nitrobenzoic acid).

15 22. A pharmaceutical composition that inhibits viral growth or replication, which composition comprises a sulfhydryl-reactive compound and a pharmaceutically acceptable carrier.

20 23. The composition of claim 22, wherein said sulfhydryl-reactive compound is selected from the group consisting of 5,5'-dithio-bis(2-nitrobenzoic acid) and pharmaceutically acceptable salts thereof.

24. The composition of claim 23, wherein said sulfhydryl-reactive compound is 5,5'-dithio-bis(2-nitrobenzoic acid).

25. A method of inhibiting the growth or replication of a virus by reaction with a protease for said virus, which method comprises contacting a virus with a composition comprising a compound that reacts with an amino acid on an exposed surface of the viral protease.

26. The method of claim 25, wherein said exposed surface is outside of the active site of said viral protease.

27. The method of claim 26, wherein said virus is a retrovirus.

28. The method of claim 27, wherein said virus is a human immunodeficiency virus.

29. The method of claim 28, wherein said human immunodeficiency virus is HIV-1.

30. The method of claim 28, wherein said compound reacts with amino acid-67.

31. The method of claim 29, wherein said compound reacts with cysteine-67.

32. The method of claim 31, wherein said compound is a sulfhydryl-reactive compound.

33. The method of claim 32, wherein said sulfhydryl-reactive compound is selected from the group

consisting of 5,5'-dithio-bis(2-nitrobenzoic acid) and pharmaceutically acceptable salts thereof.

34. The method of claim 33, wherein said
5 sulfhydryl-reactive compound is 5,5'-dithio-bis(2-nitrobenzoic acid).

35. A method of treating AIDS in a human infected
with HIV, which method comprises administering to the
human a therapeutically effective dose of a composition
comprising a compound that reacts with an amino acid on
10 an exposed surface of a protease that is encoded by the
HIV.

36. A method according to claim 35, wherein said
amino acid is amino acid-67.

37. A method according to claim 36, wherein said
15 HIV is HIV-1.

38. A method according to claim 37, wherein said
amino acid-67 is cysteine.

39. A method according to claim 35, wherein said
compound is a sulfhydryl-reactive compound.

20 40. A method according to claim 39, wherein said
sulfhydryl-reactive compound is selected from the group
consisting of 5,5'-dithio-bis(2-nitrobenzoic acid) and
pharmaceutically acceptable salts thereof.

41. The method of claim 40, wherein said
sulfhydryl-reactive compound is 5,5'-dithio-bis(2-
nitrobenzoic acid).

42. A method of prophylactic treatment of a human
5 at risk for HIV infection, which method comprises
administering to the human a prophylactic effective
dose of a pharmaceutical composition comprising a
compound that is capable of reacting with an amino acid
on an exposed surface of a protease encoded by HIV.

10 43. The method of claim 42, wherein said compound
is a sulfhydryl-reactive compound.

44. A method according to claim 43, wherein said
sulfhydryl-reactive compound is selected from the group
consisting of 5,5'-dithio-bis(2-nitrobenzoic acid) and
15 pharmaceutically acceptable salts thereof.

45. The method of claim 44, wherein said compound
is 5,5'-dithio-bis(2-nitrobenzoic acid).

46. A method of inhibiting a protease encoded by
HIV, which method comprises contacting an HIV protease
20 with a compound capable of reacting with an amino acid
on an exposed surface of said protease.

47. The method of claim 46, wherein said compound
is a sulfhydryl-reactive compound.

48. The method of claim 47, wherein said compound
25 is selected from the group consisting of 5,5'-dithio-

bis(2-nitrobenzoic acid) and pharmaceutically acceptable salts thereof.

49. The method of claim 48, wherein said compound is 5,5'-dithio-bis(2-nitrobenzoic acid).

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/00889

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.C1. 5 A61K31/19		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.C1. 5	A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (USA) vol. 88, no. 13, 1 July 1991, pages 5552 - 5556 ANDERS R. KARLSTRÖM ET AL 'COPPER INHIBITS THE PROTEASE FROM HUMAN IMMUNODEFICIENCY VIRUS 1 BY BOTH CYSTEINE-DEPENDENT AND CYSTEINE-INDEPENDENT MECHANISMS'	1-6, 9-12, 15-19, 22, 25-32, 35-39, 42-43, 46
X A	see the whole document especially p.5555, column 1, lines 46-68 ---	47 7, 20, 33, 34, 40, 41, 44, 45, 48, 49
<p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
07 MAY 1993	24. 05. 93	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	MAIR J.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		Relevant to Claim No.
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	
A	<p>SCIENCE vol. 245, 11 August 1989, pages 616 - 621 ALEXANDER WLODAWER ET AL 'CONSERVED FOLDING IN RETROVIRAL PROTEASES CRYSTAL STRUCTURE OF A SYNTHETIC HIV-1 PROTEASE' cited in the application see the whole document especially p.620, column 2, lines 3-13 ---</p>	1-49
A	<p>WO,A,9 108 769 (TRANSGENE SA) 27 June 1991 see the whole document ---</p>	1-49
P,X	<p>WO,A,9 215 329 (THE UNITED STATES OF AMERICA) 17 September 1992 see the whole document ---</p>	1-6, 9-12, 15-19, 22, 25-32, 35-39, 42,43,46 47
X	<p>KOREAN BIOCHEMICAL JOURNAL vol. 19, no. 3, 1986, pages 287 - 293 CHOI B.B. ET AL 'REVERSIBLE INACTIVATION OF SERRATIA PROTEASE BY 5,5'-DITHIOBIS (2-NITROBENZOATE)' cited in the application see page 288, column 2, line 30 - page 288, column 2, line 42 -----</p>	22-24

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/00889

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-21,25-49 are drafted as methods of treatment of the human body, the search has been carried out and directed towards the alleged effects of the compounds.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9300889
SA 70037

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

07/05/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9108769	27-06-91	FR-A- 2668065	24-04-92
		AU-A- 6975591	18-07-91
		EP-A- 0506769	07-10-92
WO-A-9215329	17-09-92	AU-A- 1463992	06-10-92